SUPPORT FOR THE AMENDMENTS

Claims 3 and 4 have been amended.

Support for the amendment of Claims 3 and 4 is provided by page 21, lines 12-21.

No new matter has been added by the present amendment.

REMARKS

Claims 1-34 are pending in the present application.

The rejection of Claims 3-4 and dependent claims 5-33 and 34 under 35 U.S.C. §112, first paragraph (enablement), is obviated by amendment.

The Examiner has taken the position that the recited stringency in previously pending Claims 3 and 4 (washing at 60°C and a salt concentration equivalent to 0.1 x SSC and 0.1% SDS) is "medium" stringency and would allow for hybridization of DNA having 80% to 90% identity. Applicants note that the Examiner offers no support for this allegation and, as such, has not shifted the burden upon Applicants to prove otherwise as asserted.

Nonetheless, in order to expedite examination of this application, Applicants have amended Claims 3 and 4 based on page 21, lines 12-21 of the specification to be 65°C and 0.1xSSC and 0.1% SDS. Applicants submit that such stringent conditions are sufficient to enable the skilled artisan practice the claimed invention without undue experimentation.

With respect to the scope of homologs expected to be identified by hybridization conditions defined in Claims 3 and 4, Applicants **submit herewith** a general information guide on hybridization conditions that is available at: http://www.ndsu.nodak.edu/instruct/mcclean/plsc731/dna/dna6.htm (see, for example, page 5 under the heading "Stringent Wash"). From this general information guide Applicants submit that would be well within the understanding of the skilled artisan that the claimed hybridization conditions are very stringent so as to only to permit the identification of homologs having a very high degree of homology. Applicants further submit that it is well within the purview of the skilled artisan to make and assess this very limited number of homologs for their standing with respect to the claimed invention without undue experimentation.

In view of the foregoing amendment and the general state of the art, Applicants submit that the claimed invention is in full compliance with the enablement requirement of 35 U.S.C. §112, first paragraph.

Applicants request withdrawal of these grounds of rejection.

Applicants submit that the present application is now in condition for allowance. Early notification of such action is earnestly solicited.

Respectfully submitted,

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DNA - Basics of Structure and Analysis

Nucleic Acid Hybridizations

The hybridization of a radioactive probe to filter bound DNA or RNA is one of the most informative experiments that is performed in molecular genetics. Two basic types of hybridizations are possible.

Southern hybridization - hybridization of a probe to filter bound DNA; the DNA is typically transferred to the filter from a gel

Northern hybridization - hybridization of a probe to filter bound RNA; the RNA is typically transferred to the filter from a gel

Probes are the primary tool used to identify complementary sequences of interest. Generally, the probe is a clone developed by inserting DNA into a vector. Most often these are plasmid clones.

Probe - a single-stranded nucleic acid that has been radiolabelled and is used to identify a complimentary nucleic acid sequence that is membrane bound

The hyrbridization process involves two different steps. First the nucleic acid must be immobilized on a filter. This is generally called a "Southern Transfer" procedure. The second step is the actual hybridization of the probe to the filter bound nucleic acid.

The following steps describe the Southern transfer procedure.

1. Digest DNA with the restriction enzyme of choice.

2. Load the digestion onto a agarose gel and apply an electrical current. DNA is negatively charged so it migrates toward the "+" pole. The distance a specific fragment migrates is inversely proportional to the fragment size.

3. Stain the gel with EtBr, a fluorescent dye which intercalates into the DNA molecule. The DNA can be visualized with a UV light source to assess the completeness of the digestion.

4. Denature the double-stranded fragments by soaking the gel in alkali (>0.4 M NaOH).

5. Transfer the DNA to a filter membrane (nylon or nitrocellulose) by capillary action. Typically a Southern transferst setup contains (from bottom to top):

    - buffer
    - sponge
    - filter paper
    - the gel containing the nucleic acid
    - a nylon or nitrocellulose membrane
    - more filter paper
    - paper towels to catch the buffer that passed through all of the above

Southern hybridizations with plant DNA is not a trivial matter. The primary requirement for a successful experiment is that the DNA to be probed is digested to completion. We have already discussed the choice of enzymes in this regard. Even when using compatible enzymes (not GC or GXC sensitive) monitoring the completeness of the reaction is essential for consistent results.

Once you are satisfied that you completely digested the DNA and are confident that it was successfully transferred to the filter membrane, the next step is perform the actual hybridization. The following steps describe the procedure.

Steps in Southern Hybridization Procedure

1. Prepare a probe by nick translation or random, oligo-primed labelling.

2. Add the probe to a filter (nylon or nitrocellulose) to which single-stranded nucleic acids are bound. (The filter is protected with a prehybridization solution which contains molecules which fill in the spots on the filter where the nucleic acid has not bound.

3. Hybridize the single-stranded probe to the filter-bound nucleic acid for 24 hr. The probe will bind to complementary sequences.

4. Wash the filter to remove non-specifically bound probe.

5. Expose the filter and determine:
   - **a. Did binding occur?**
   - **b. If so, what is the size of hybridizing fragment?**

Hybridization Stringency

The temperature and salt concentrations at which we perform a hybridization has a direct effect upon the results that are obtained. Specifically, you can set the conditions up so that your hybridizations only occur between the probe and a filter bound nucleic acid that is highly homologous to that probe. You can also adjust the conditions the hybridzation is to a nucleic acid that has a lower degree of homology to the probe.

Your hybridization results are directly related to the number of degrees below the Tm of DNA at which the experiment is performed. For a aqueous solution of DNA (no salt) the formula for Tm is:

T_m = 69.3°C + 0.41(% G + C)°C

From this formula you can see that the GC content has a direct effect on Tm. The following examples, demonstrate the point.

T_m = 69.3°C + 0.41(45)°C = 87.5°C (for wheat germ)

T_m = 69.3°C + 0.41(40)°C = 85.7°C

T_m = 69.3°C + 0.41(60)°C = 93.9°C

Hybridizations though are always performed with salt. This requires another formula which that takes the salt concentration into account. Under salt-containing hybridization conditions, the effective Tm is what controls the degeree of homology between the probe and the filter bound DNA is required for successful hybridization. The formula for the Effective Tm (Eff T_m).

Eff T_m = 81.5 + 16.6(log M [Na+]) + 0.41(%G+C) - 0.72(% formamide)

The salt solution that is most often used in hybridization experiments is SSC (standard sodium citrate). Different concentrations of this solution are used at different steps in the hybridization procedure. The following table gives

the Na+ concentration for different strengths of SSC. Remember that this value is essential to derive the Eff T_m.

Na+ ion concentration of different strengthsof SSC

SSC Content	[Na+] M
20X	3.3000
10X	1.6500
5X	0.8250
2X	0.3300
1X	0.1650
0.1X	0.0165

Another relevant relationship is a that ***1% mismatch of two DNAs lowers the T_m 1.4°C.*** So in a hybridization with wheat germ that is performed at T_m - 20°C (=67.5°C), the two DNAs must be 85.7% homologous for the hybridization to occur. 100% - (20oC/1.4°C) = 85.7% homology

Let's now look at an actual experiment, the hybridization of a probe with filter bound wheat DNA in 5X SSC at 65oC. The first step is to derive the Eff T_m.

Eff T_m = 81.5 + 16.6(log 0.825) + 18.5 = 98.6°C

These types of hybridization experiments are typically performed at T_m - 20°C. A typical temperature of hybridization is 65°C. (If formamide is used the hybridization is normally performed at 42°C). With these conditions, 83.1% homology between the probe and filter bound DNA is required for hybridization. The following calculation is how this number was derived.

100 - [(98.6-65.0)/1.4] = 100 - (23.6/1.4) = 83.1%.

The next step in a hybridization experiment is to wash the filter. This is normally done in two steps. First a non-stringent wash is performed to remove the non-specifically bound DNA and the second wash is performed at a higher stringency that only permits highly homologous sequences to remain bound to the filter. Controlling the stringency is an important step in these experiments.

Stringency - a term used in hybridization experiments to denote the degree of homology between the probe and the filter bound nucleic acid; the higher the stringency, the higher percent homology between the probe and filter bound nucleic acid

Non-stringent wash: normally 2X SSC, 65°C

Eff T_m = 81.5 + 16.6[log(0.33)] + 0.41(45%)= 92.0°C

%Homology = 100 - [(92-65)/1.4] = 80.7%

Stringent wash: normally 0.1X SSC, 65°C

Eff T_m = 81.5 + 16.6[log(0.0165)] + 0.41(45%) = 70.4°C

%Homology = 100 -[(70.4-65)/1.4] = 96.1%

This example shows that the final wash is the one of concern when determining the relatedness of the probe and the filter bound nucleic acid.

An example: Bowman-Kirk Protease Inhibitor Final wash is performed at 0.2X SSC, 55oC; assume 45% GCcontent

Eff T_m = 81.5 + 16.6[log(0.033)] + 18.5 = 75.4°C

%Homology = 100 - [(75.4-55.0)/1.4] = 85.4%

The point to this last example to emphasize that your percent homology is directly related to your most stringent condition in your hybridization experiment. This invariably is the final wash. Thus, you only need to make this calculation to determine the stringency of your experiment.

What You Can Learn from Southern Hybridizations

Southern hybridizations have many applications. The first application after cloning a gene is often to determine how many copies of the gene are in the species from which the gene was cloned. This experiment is performed by hybridizing a clone of the gene to total DNA that has been digested with several enzymes. The procedure is termed a ***genomic southern.***

One gene that has drawn intense interest because of its potential applied usage in plant biotechnology is chitinase.

We have already discussed the isolation of a clone for this gene from bean. As you can imagine, the gene has also been cloned from other species. The first page of hybridization handout shows the southern hybridization pattern obtained from cucumber, rice and bean. These hybridizations show that these species contain ***different copy numbers*** for the gene.

A second application for southern hybridizations is the estimation of copy number of a specific gene. This experiment is performed by running several lanes with different copy numbers of the gene to which you are probing and comparing the hybridization intensities with a companion genomic southern experiment. This is called a ***reconstruction experiment.*** The example in on the second page of the handout is for phaseolin, the major storage protein of bean. In this example, 1, 2 and 5 genomic equivalents (copies per bean genome) are seen in lanes 7-9. The other lanes are various restriction digestions of total bean DNA. After hybridization, densitometric readings were taken and it was determined that bean contained 6.5 copies of the gene. This agreed with the data obtained from reassociation kinetic experiments.

Southern hybridization analysis can also be performed to determine if a phenotypic mutation is due to a structural change in the gene controlling the trait of interest. If a gene undergoes an insertion or deletion the resulting hybridization pattern would be changed. Insertional mutagenesis would generate fragments of an increased size whereas deletions would reduce the size of the hybridizing band. Two tomato mutants, Neverripe (nr) and ripening inhibitor (ri) express polygalacturonase, an enzyme involved in fruit ripening, at lower levels than normal or wild type tomato. The question posed here was whether the structure of the polygalacturonase (and other ripening specific genes) are structurally different than the wild type genotype. The thrid page of the handout. shows that the structure of these genes in the mutatnt is not different than the wild type. Therefore, some other molecular event is responsible for these differences in expression patterns of the different mutants.